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Author(s)	Okada, Yoshio; Nishida, Seishu; Tadokoro, Jun
Citation	Biken's journal : journal of the Research Institute for Microbial Diseases. 1961, 4(3), p. 209-213
Version Type	VoR
URL	https://doi.org/10.18910/83053
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BIKEN'S JOURNAL 4, 209-213 (1961)

Correlation between the Hemagglutination Titer and the Virus Particle Number of HVJ

YOSHIO OKADA, SEISHU NISHIDA* AND JUN TADOKORO**

Department of Preventive Medicine, The Research Institute for Microbial Diseases, Osaka University, Osaka
(Received for publication, September 6, 1961)

SUMMARY

Data on the particle count by both the red blood cell adsorption technique and the hemagglutination titer by Salk's pattern method of purified samples of HVJ were compared. Thus the relationship between the hemagglutinating unit and the equivalent virus particle number was found. One Salk's HA unit of this virus corresponded to 2.4×10^7/ml when a standard concentration of red blood cells of 2.57×10^7/ml was used in the titration. The ratio between these values is almost unity and this fact indicates that in Salk's pattern method the virus particle number and the number of red blood cells used for the titration is equal in the tube showing the end point in which the minimal conditions for complete hemagglutination are satisfied.

The occurrence of a positive pattern in Salk's method is discussed.

INTRODUCTION

Various properties of HVJ were studied by Fukai and Suzuki (1955) and the antigenic architecture of the virus was studied by Hosaka *et al.* (1960). The modified particle formation in undiluted passages of HVJ were reported by Tadokoro (1958), while Okada *et al.* (1957) described fused giant cell formation by the virus in Ehrlich's tumor cells.

These observations are closely related and cannot fully be explained except in relation to each other. Fortunately all these observations have a common parameter, the hemagglutination titer for the amount of virus, because the hemagglutinating activity is determined by a simple technique and is more stable than other biological activities of HVJ.

This study was a fundamental analysis for the full understanding of above mentioned data. The virus particle number in one HA unit of virus was measured using the red cell adsorption technique (Donald and Isaacs, 1954; Isaacs and Donald, 1955) and Salk's pattern method under standard conditions.

Present address:

* Radiation Center of Osaka Prefecture

** Public Health Research Institute, Osaka Prefecture

MATERIALS AND METHODS

1. *Virus*

HVJ, Z strain, which had been isolated from mice in our laboratory(Fukai and Suzuki, 1955), was used. The seed chorioallantoic fluid was diluted to 10^{-6} with broth. 0.2 ml aliquots of the diluted inoculum were injected allantoically into 10-day-old embryonated eggs. After 72 hours incubation at 35.5°C, the infected fluid was harvested and cetrifuged at 34,850 × g, for 30 minutes in a Spinco L No. 30 roter after low speed centrifugation at 1,400 × *g* for 20 minutes to eliminate cell debris. The resulting pellet was resuspended in M/100 phosphate buffered saline(PBS) at pH 7.2 and cetrifuged again at 1,400 × *g* for 20 minutes to eliminate coarse aggregates.

The purified sample was diluted to desired concentrations and used for the virus assay.

2. *Red blood cells*

a) *Cells for measuring the hamagglutination titration.* Fowl red blood cells, withdrawm into sodium citrate, were washed 3 times with PBS and adjusted with PBS to a concentration of 2.57×10^7/ml. This concetration corresponded to a 0.5 per cent suspension of packed red cells. It was used for measuring the hemagglutination titer.

b) *Cells for measuring the particle count.* Two tenth ml of 5 per cent saponin in saline was added to 10 ml of a 10 per cent suspension of washed red blood cells at room temperature. After 5 minutes the sample was washed 4 times with PBS. Then the cells were treated with M/1000 KIO_4 in PBS for 30 minutes at room temperature. After centrifugation at 500 × *g* for 10 minutes, the sediment was resuspended in PBS containing 5 per cent glucose and incubated in ice water for 30 minutes. The sample was further washed with, and resuspended in PBS at concentration of 5.5×10^8 or 1.2×10^9/ml.

3. *Hemagglutination titration*

Salk's pattern method was used. Serial two fold dilutions of the virus were made in 0.5 ml of PBS, and an equal volume of 2.57×10^7/ml of fowl red blood cells was added. After incubation at room temperature for 100 minutes, the titer was determined taking the last tube showing so-called "complete agglutination" (complete adhesion of red cells onto the concave bottom surface of the tube) as the end point. The titers were then calculated as titers per ml.

EXPERIMENTAL

One ml of the cell suspensions for particle counts (containing 5.5×10^8 or 1.2×10^9 red blood cells) and an equal volume of the virus sample were mixed as shown in Table 1, and kept in ice water for 60 minutes. After centrifuged at 500 × *g* for 10 minutes the unadsorbed virus hemaglutinin titer in the supernatant fluid was always less than 5 per cent of the added virus and this small amount of non-adsorbed virus was neglected in following calculations. It is interesting that the red cell ghosts were scarcely agglutinated, though the ghosts were given enough to be agglutinated and virus particles were adsorbed in a sufficient amount to evoke the agglutination.

The cells which adsorbed virus were fixed with 0.1 per cent buffered osmic acid for 60 minutes at 4°C and washed with distilled water 3 times. They were dried on collodion covered grids for electron microscopic examination.

A cell which had adsorbed virus is shown in Fig. 1. Fifty cells from each sample

were photographed. To obtain the number of particles adsorbed onto the red blood cells, the number of particles on the transparent part of the cell (N_t) was counted from the photographs. Then the photographs of the cells were cut out from enlargments and the nulcear and cytoplasmic parts were separated and weighed to determine the areas of these two parts. As the nuclei of the cells are opaque, the presumptive virus particle number adsorbed onto the cell surface masked by the shade of nuclei(N_n) must be added to the number N_t counted in the cytoplasm (transparent) area. The mean area of nucleus projection thus obtained was 33 per cent of the whole cell projection. An estimation of the number of virus particles adsorbed per cell N is thus:

$N=N_t+N_n=1.5\ N_t$.

Table 1.

No. of experiment	Red blood cell concentration used ($\times 10^8$/ml)	Virus titer added (HAU/ml)	Mean virus particle number adsorbed onto one red blood cell	Virus particle number per one HAU, calculated ($\times 10^7$)
1	5.5	3,072	61	1.09
2	5.5	1,536	32	1.15
3	12.0	2,048	57	3.34
4	12.0	2,048	62	3.60
5	12.0	4,600	102	2.65
6	12.0	4,600	104	2.70
mean				2.42 ± 0.49

As can be seen in Table 1, the hemagglutination titration and the particle count were carried out simultaneously with 6 different samples. The mean value for the virus particle number per one HA unit was $(2.42\pm0.49)\times 10^7$, ($p=0.05$). As 2.57×10^7/ml red blood cells was used for measurement of the hemagglutination titration and the HA titer is expressed per ml of the sample in these experiments, the result shows that when Salk's pattern method is used under standard conditions the fowl red blood cell number is equal to the virus particle number in the last tube giving so-called complete agglutination. Namely the virus particle number in the end point tube can be expressed by the number of red blood cells used.

DISCUSSION

Donald and Isaacs(1954), Isaacs and Donald(1955) and Fukai(1961) have reported similar results to ours in Influenza A, Influenza B and Newcastle disease viruses.

From the above results, it seems that the appearance of a positive pattern is due to dimer or polymer formation of red cells and if the formation is low or negligible, a negative pattern appears. There are, however, some cases in which the mechanism cannot be explained by simple polymer formation of red blood cells.

Granoff and Henle(1954) reported that in Salk's pattern method, extension of the positive region of Newcastle disease virus occurred when samples of the virus containing the same number of fowl red blood cells were repeatedly settled at 37°C and 4°C. The extension of the titer was 16 fold that of the original sample which had not been repeatedly settled. This indicates that the tube in which the red cell number was 16 fold that of the virus particles still showed a positive pattern. Fauconnier and Barua(1959) found that when mouse red cells treated with formalin were used in Salk's pattern method instead of normal mouse red cells, the hemagglutination titer of Influenza viruses increased. They suggested that this might be due to a lowering of the cell surface charge by formalin treatment. Hanig (1948) observed that when human red cells had been in contact with Influenza PR8 virus at 22°C, the mobility of the negative charge of the red cells was irreversively reduced to less than half the untreated value of 1.3 (μ/sec.)/(volt/cm). Furthermore it has been observed from an analysis of the hemolytic reaction of HVJ that the surface of fowl red blood cells was critically affected by one HVJ particle adsorption only (Okada).

The occurrence of positive and negative patterns on Salk's pattern method may be explained as follows: non virus adsorbed red blood cells slip down along the concave bottom surface of the tube to the center. However red blood cells which have adsorbed viruses such as Influenza, NDV or HVJ, suffer some physical changes in their surface such as a reduction in their repellent charge. Thus these red cells have a tendency to adhere to the wall of the tube and a positive pattern appears. This tendency may be reflected by the agglutination of the cells participating in the reaction in the virus-cell mixture.

The ratio of the infectivity titer to the hemagglutination titer of fresh HVJ-infected allantoic fluids were 10^6 to 5×10^6 as reported by Tadokoro (1958). Calculating from this value and our results, that one HA unit is equal to 2.4×10^7 particles, shows that for HVJ, one infective dose consists of 5 or more (5 to 24) virus particles.

The authors wish to thank Prof. Konosuke Fukai for his advice. Thanks are also due to Miss Kazuko Kawai for technical aassistance.

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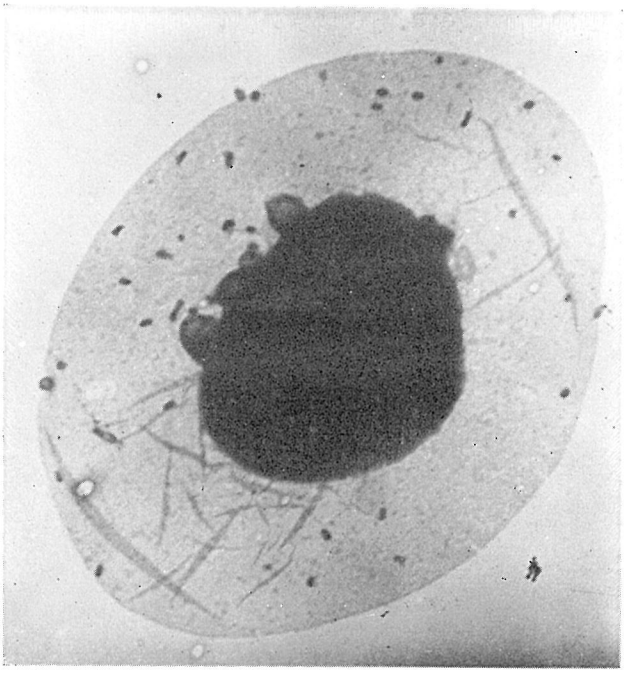

Fig. 1. Electron Micrograph of HVJ-adsorbed Fowl Red Blood Cell